



Evaluation of antidiabetic, antioxidant and antilipidemic potential of natural dietary product prepared from *Cyphostemma digitatum* in rats' model of diabetes

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ABSTRACT

Introduction: *Cyphostemma digitatum* has a high content of antioxidant constituents and has been employed by the traditional healers and local people of Yemen for diabetes treatment. However, scientific evidence regarding its antidiabetic efficacy is largely unknown. Accordingly, the present study aimed to confirm the treatment effects of a dietary natural product prepared from *Cyphostemma digitatum* (PCD) in diabetic rats.

Methods: Diabetes was induced by a high-fat diet and streptozotocin (HF-STZ). PCD (1 g/kg) was given by gavage administration once a day continuously for 30 days. At the end of treatment, blood and skeletal muscle samples were collected for further analysis.

Results: The antidiabetic effects of PCD were demonstrated by significant reduction ($P \leq 0.05$) in the levels of serum glucose (40%), triglyceride (32%), cholesterol (53%), low-density lipoprotein (LDL) (44%), malondialdehyde (MDA) (61%) in PCD treated groups compared to the diabetic control group. Additionally, PCD treatment significantly ($P \leq 0.05$) restored the decreased levels of insulin (70%) and the activities of superoxide dismutase (SOD) (57%) and reduced glutathione (GSH) (544%) when compared to that of diabetic control rats. We found that treatment with PCD for 30 days fully restored the plasmalemmal glucose transporter type 4 (GLUT4) contents, as well as the phosphorylation of phosphatidylinositol 3-kinase (PI3K) ($P \leq 0.05$).

Conclusion: Thus, PCD treatment can be considered a potential drug candidate for diabetes.

Implication for health policy/practice/research/medical education:

Cyphostemma digitatum is an alternative phytomedicine endowed with antidiabetic potentials. The mechanism probably involves its high antioxidant activity. This research shows the promising antidiabetic prospects of *C. digitatum* in rats. Thus, this plant might be considered a candidate for developing drugs against diabetes.

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Introduction

Diabetes is a disease of chronic hyperglycemia, which occurs when the body has lost its ability to produce insulin or when insulin cannot exert its full effect due to resistance to its action or a combination of both (1).

In muscle cells, two major signaling pathways are involved in glucose uptake, one is promoted by insulin through phosphatidylinositol 3-kinase (PI3K); the other by the activation of AMP-activated protein kinase (AMPK) (2). Both pathways promote the translocation of glucose transporter type 4 (GLUT4), the principal

glucose transporter protein, from intracellular pools into sarcolemma of the muscle cells (2). Therefore, the correct functioning of the PI3K and AMPK pathways is essential for proper metabolic control of glucose, and their dysfunction impairs glucose homeostasis.

Although the etiology of type 2 diabetes is poorly defined, numerous studies have demonstrated that oxidative stress has a role in affecting insulin sensitivity and/or β -cell mass (3). Hence, therapy with antioxidants may signify a useful pharmacologic overture to the management of diabetes.

In various parts of the world, there has been an evolving

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interest in studying the medicinal importance of plants and herbs. This interest is widely attributed to the fact that traditional plant and herb constituents could be a source of various phytochemicals endowed with antioxidant potential (4).

Yemen or Queen of Sheba ethnobotany resembled the richest botanical culture in the Middle East and North Africa; for example, this ethnobotany's influences on Chinese medicine have been reported (5). One of the recently increasingly reported plants in literature from Yemen ethnobotany is *Cyphostemma digitatum* (Vitaceae). The local name is Halqa; the first report about this plant's ecology, preparation, and application as a culinary and medicinal plant was in one of the oldest books about medicinal plants in Yemen in 1294. *C. digitatum* is used mainly as a food flavoring, especially as the main constituent of traditional Yemeni soup (Marak), and in general health support (6). *C. digitatum* is reported to be useful in providing relief from vomiting and asthma (7), and it has also been described as a medication for gastroenteritis, fatigue, headache, and malaria (6).

The current method of preparation and storage of the traditionally processed dietary product (used in this research) was reported by Al-Duais et al (8), who showed that *C. digitatum* had a high antioxidant capacity and high total phenolics by different assays (8). Consistent with this, others found that *C. digitatum* exhibits antioxidant action (9). It is well supported in the literature that plants and herbs with high antioxidant activities are suggested as better therapeutic agents in helping prevent a wide range of diseases, including type 2 diabetes, heart disease, and cancer (10,11). *C. digitatum*, after processing, is also confirmed to have a high content of vitamin A & C, carotenoids, tocopherols, and tocotrienols, some of which rarely exist in nature isomers (6).

Although *C. digitatum* potentially has beneficial health properties (6,9) and contains a substantial quantity of phytopolyphenolics, known for its important antioxidant activity (8,9), it is also employed by the traditional healers and local people of Yemen for diabetes treatment. However, reports concerning its effect on insulin sensitivity and oxidative stress are not available in the literature. Therefore, the current study was designed to investigate the impact of the natural dietary product prepared from *C. digitatum* (PCD) on the effective management of diabetes through antioxidant defense mechanism in a model of type 2 diabetes.

Materials and Methods

Chemicals and Reagents

All fine chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) and antibodies from a variety of sources. PCD samples were given as a gift from Dr. Al-Duais.

Plant materials

The leaves and young branches of *C. digitatum* were

collected from a natural area in Baddan Mountain in Yemen, away from any anthropogenic effect. The plant was identified and authenticated by Dr Al-Duais; a voucher sample was prepared and deposited in the herbarium at Ibb University for future reference (275CD).

Preparation of PCD

PCD was prepared according to Al-Duais et al (8). Briefly, about 600 g of the leaves and young branches of *C. digitatum* were boiled in 1000 mL distilled water for 30 minutes under pressure. Subsequently, the resultant gummy material of leaves and young branches mass was mixed with a wooden spoon, and the water was removed by pressing through a cotton bag. The thick homogeneous paste was thinned into disks (8–12 cm in diameter) and dried in the sun on clean plates covered with tiny mesh, turned upside down each day until reaching complete dryness. These samples were packed in plastic bags and stored at ambient temperature (30°C) for 3–5 months before being used. From this dry sample, a fresh stock for daily use was prepared.

Animals

Male Wister rats weighing between 150 and 180 g were obtained from the animal house at Yarmouk University and housed under good hygienic conditions of $23 \pm 2^\circ\text{C}$ with 12 hours light/12 hours dark cycles. The animals consumed a normal laboratory diet, and water was provided *ad libitum*. All experimental procedures were carried out following the current ethical and care guidelines for the care of laboratory animals and were approved by the Animal Ethics Committee of Yarmouk University, Irbid, Jordan.

Acute toxicity studies

Lethal dose (LD50) of PCD was estimated in mice according to the protocol described by Lorke (12). Mice were randomly assigned to different groups containing 7 mice in each group. PCD (10, 100, 1000, 2000, and 3000 mg/kg) was administered orally (10 mg/mL distilled water) to six groups of mice. The mice were allowed food and water *ad libitum*. Signs of toxicity and mortality were recorded within 72 hours.

Induction of diabetes in the rats

Diabetes was induced by feeding the experimental rats with a high fat diet (HF) (45% fat) for 30 days, followed by a single intraperitoneal injection of 40 mg/kg STZ freshly dissolved in cold citrate buffer (0.1 M, pH 4.5). Normal control animals were injected with the citrate buffer vehicle. The rats were confirmed diabetic by the elevated plasma glucose levels after 72 hours of injection. The rats with stable hyperglycemia (blood glucose >200 mg/dL) were used for the experiment. The day on which hyperglycemia was confirmed designated as day 0. Negative control rats received a normal pellet diet.

Experimental Design and Tissue Collection

The rats were randomly divided into four groups ($n=7$ each) as follows: normal control group (non-diabetic, ND) was administered with 2 mL/kg/d of distilled water; diabetic group (D) was administered 2 mL/kg/day of distilled water; diabetic group was treated with 1 g/kg PCD (D + PCD); and a diabetic group was treated with 200 mg/kg metformin (D + MET).

All treatments were given orally, once per day. At the end of 30 days of treatment, the rats were anesthetized with ether, and blood and skeletal muscle samples were collected for further analysis.

Biochemical investigations

After 30 days of PCD administration, the rats were fasted overnight and euthanized under ether, and their blood was collected and centrifuged at 1400 g for 10 minutes to obtain serum. Blood glucose levels were determined using a glucometer (ACCU-CHECK Active kit, Roche Diagnostics, Mannheim, Germany). Fasting serum insulin levels were determined as described previously (13). Total cholesterol (TC), triglyceride (TG), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) were measured using commercially available kits (linear chemicals SL, Barcelona, Spain) according to the manufacturer's instructions.

Detection of oxidative stress indicators

Reduced glutathione (GSH) and superoxide dismutase (SOD) were measured in the serum using assay kits following the manufacturer's instructions. Lipid peroxidation levels were measured by the thiobarbituric acid reacting with the serum malondialdehyde (MDA) (14).

Plasma membrane preparation

Plasma membranes were obtained from giant sarcolemmal vesicles, as has previously been reported (15). Briefly, skeletal muscles were cut into thin layers (~1–3 mm thick) with a scalpel. The scored muscles were then incubated for 75 minutes at 34°C in 140 mM KCl-10 mM MOPS (pH 7.4), collagenase (150 U/mL), and aprotinin (1 mg/mL). After that, the incubating medium was collected, and the remaining muscle debris was washed with 10 mM EDTA in KCl/MOPS until 7 mL. Percoll (final concentration 16%) and aprotinin (1 mg/mL) were added to the collected medium. The resulting mixture was placed at the bottom of a density gradient consisting of a 3-mL middle layer of 4% Nycodenz (wt/vol) and a 1-mL KCl-MOPS upper layer. The samples were spun at 60 g for 45 minutes at room temperature. After centrifugation, the vesicles were harvested from the interface of the two upper solutions and centrifuged at 12000 g for 5 minutes. The supernatant fraction was aspirated, and the resulting pellet was resuspended in KCl/MOPS. Vesicles were stored at –80°C for subsequent analysis.

Western blot analysis

Western blot analysis was performed as previously described (2,15). Briefly, skeletal muscle was homogenized in 2 mL homogenizing buffer. After homogenization, the solution was sonicated (5 seconds) and set to rock end over end for 30 minutes at 4°C. The solution was centrifuged at 1500 g for 15 minutes at 4°C, and the supernatant was collected. Protein samples were subjected to SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes, which were then incubated for 2 hours with a blocking solution and then incubated overnight with the PI3K-P and GLUT4 primary antibodies (Sigma-Aldrich, St. Louis, USA). The blots were washed three times with washing buffer (Tween-20/Tris-buffered saline), followed by incubation with goat anti-rabbit secondary antibodies (Invetrogen-Thermo Fisher, Rockford, USA) overnight at room temperature. Then, the blots were washed three times, and the immune reactive protein bands were visualized with diaminobenzidine substrate. The protein band intensity was measured relative to the control group using the ImageJ software (NIH, Bethesda, MD).

Statistical analysis

All data are expressed as the mean \pm SEM. Differences between groups were calculated by one-way analysis of variance (ANOVA) using SPSS software (SPSS Inc., Chicago, IL). The significant value of difference was considered when the P value < 0.05 .

Results

Acute toxicity

It was not possible to obtain the LD50 of PCD, because administering mice with increasing doses of PCD up to 3000 mg/kg showed neither signs of morbidity nor behavioral changes. It indicates that PCD was highly safe and nontoxic in rats up to the oral dose of 3000 mg/kg body weight. We, therefore, carried out our investigations with 1/3th maximum dose of PCD that is 1000 mg/kg.

Effect of PCD on serum glucose and insulin levels

Compared with ND, blood glucose was significantly higher in the D group ($P < 0.05$; Figure 1A). Administration of PCD or MET for 30 days significantly lowered blood glucose levels ($P < 0.05$; Figure 1A) but not for those observed in the ND group ($P > 0.05$; Figure 1A). Concurrently, circulating insulin concentrations were significantly lower in the D group than in the ND group ($P < 0.05$; Figure 1). Oral administration of PCD or MET partially restored serum insulin levels. However, it failed to restore it to the control values (Figure 1B).

Effect of PCD on plasmalemmal GLUT4 content and on PI3K phosphorylation levels

GLUT4 and PI3K phosphorylation plasmalemma content were significantly ($P < 0.05$; Figure 2A and B) lower in D than ND. Interestingly, treatment with

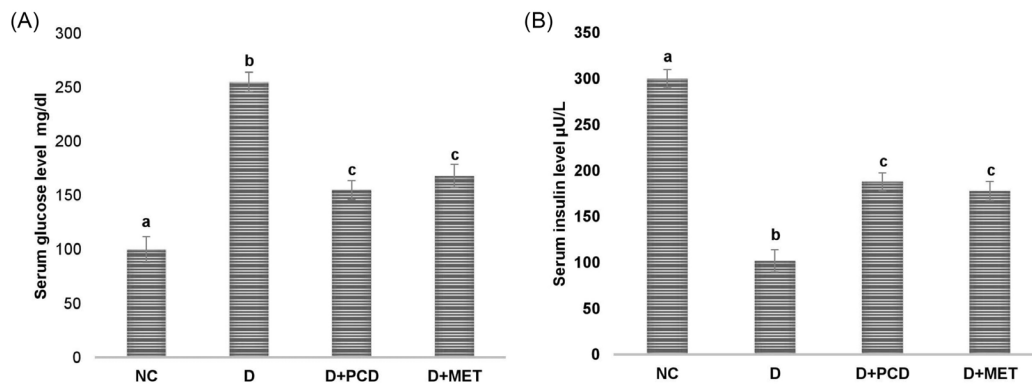


Figure 1. Effect of dietary natural product prepared from *Cyphostemma digitatum* (PCD) treatment on blood glucose (A), and serum insulin (B) levels in HF-STZ-induced diabetic rats. Data are presented as means \pm SEM; $n = 7$. Means with different superscript letters are significantly different from one another ($P < 0.05$). Abbreviations: NC, normal control; D, diabetic; D+PCD, diabetic +1 g/kg dietary natural product prepared from *Cyphostemma digitatum*; D+MET, diabetic+200 mg/kg metformin.

PCD or MET significantly ($P < 0.05$; Figure 2A and B) increased the plasmalemma content of GLUT4 and PI3K phosphorylation compared with the D group. However, PCD and MET did not induce significant restoration in GLUT4 content on the plasma membrane and PI3K phosphorylation when compared with the ND group. The total content of GLUT4 and PI3K proteins was not altered with any experimental treatments ($P > 0.05$; Figure 2A and B).

Interestingly, no significant changes were observed in the AMPK level or phosphorylation among these groups (data not shown).

Effect of PCD on lipid profiles

Diabetic rats had an aggravated blood lipid profile (elevated

TG, TC and LDL levels) (Table 1). The administration of PCD for 30 days significantly ($P < 0.05$; Table 1) reduced the TG, TC and LDL levels ($P < 0.05$) compared with the D group. The reduction in these lipid components was the same in MET treated group. Interestingly, this reduction in lipid components induced by PCD or MET administration significantly differed from the ND group. However, no significant changes were observed in serum HDL levels among these groups (data not shown). It is important to be noted that lipid profiles in all groups before treatment were the same (data not shown).

Effect of PCD on serum oxidative stress markers

We noted that serum MDA was markedly increased in the D group ($P < 0.05$; Table 2). This trend was reversed after

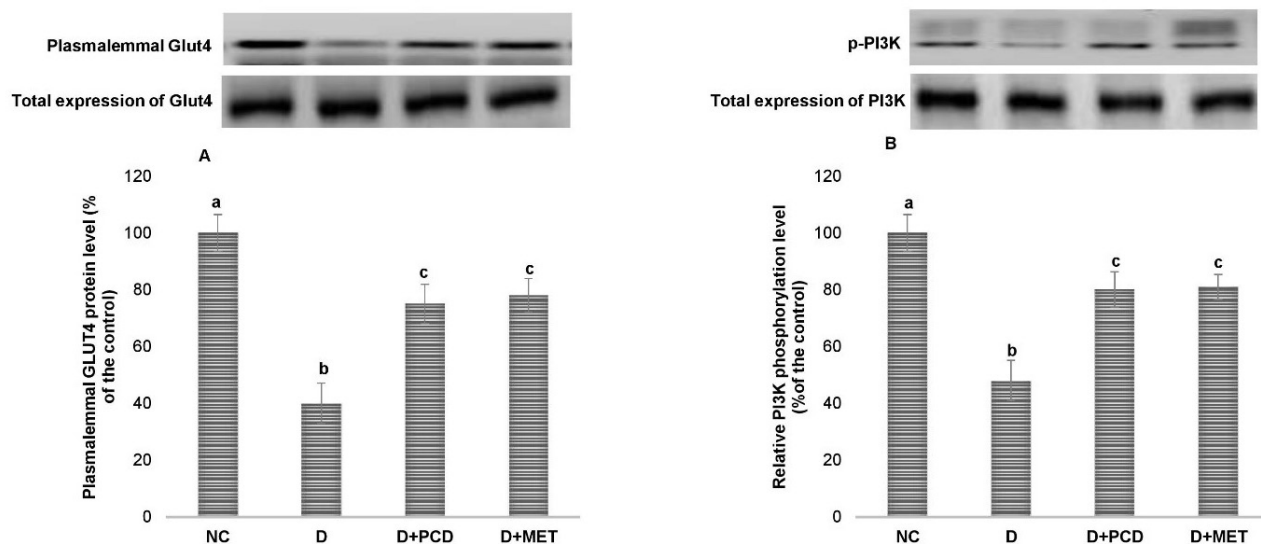


Figure 2. Effect of dietary natural product prepared from *Cyphostemma digitatum* (PCD) treatment on the plasmalemmal content of GLUT4 (A), and PI3-K phosphorylation (B) in HF-STZ-induced diabetic rats. Data are presented as means \pm SEM; $n = 7$. Equal quantities of protein were loaded for each muscle, and the loading was verified with Ponceau staining. Means with different superscript letters are significantly different from one another ($P < 0.05$). Abbreviations: NC, normal control; D, diabetic; D+PCD, diabetic +1 g/kg dietary natural product prepared from *Cyphostemma digitatum*; D+MET, diabetic+200 mg/kg metformin.

30 days of treatment by PCD or MET (Table 2). The MDA values in the D + PCD group, the D + MET group and the ND group were not significantly different ($P > 0.05$; Table 2).

A significant decrease ($P < 0.05$; Table 2) in SOD and GSH levels was found in the D group compared to the ND group. The supplementation of PCD or MET was found to significantly ($P < 0.05$) improve the levels of SOD and GSH, and these recovered levels did not show any difference between the ND group and PCD or MET administered groups ($P > 0.05$; Table 2).

Discussion

Hyperglycemia is a central problem in type 2 diabetes. Therefore, preventing and alleviating hyperglycemia is an important scenario for health promotion. Due to the side effects of currently available antidiabetic drugs (16), there is a need to look for more efficacious agents without or with fewer side effects. For this reason, it is now generally accepted that medicinal plants and herbs will be promising alternative candidates for the treatment of chronic diseases, including diabetes (15,17). Safety data obtained in the present study showed that PCD was non-toxic up to the dose of 2000 mg/kg in normal healthy mice. Therefore, 1/2 of this dose, *i.e.*, 1000 mg/kg, was selected for further detailed studies.

The data obtained in this research revealed that the rats fed HF combined with STZ exhibited the characteristic features of diabetes, namely hyperglycemia, coupled with other metabolic irregularities, including insulin resistance and hyperlipidemia. Furthermore, the present study demonstrated elevated serum lipid peroxide levels in diabetic rats and a significant decrease in the antioxidant enzymes, namely, SOD and GSH. These alterations were consistent with previous reports (18).

Our current study demonstrated for the first time that PCD has potent antidiabetic potential in HF-STZ-induced diabetic rats by (i) improving insulin secretion and/or sensitivity and stimulating skeletal muscle glucose uptake via activation of the PI3K-GLUT4 signalling pathway and by modulating, (ii) lipid profile and (iii) oxidative stress. Moreover, we found that supplementation of PCD per day for 30 days in diabetic rats showed partial restoration in serum glucose and insulin levels. It is important to note that this effect of PCD on glucose and insulin levels was comparable to the response seen with MET.

Insulin plays a pivotal role in the regulation of blood glucose homeostasis. The present study results show that PCD treatment restored the serum insulin levels in diabetic rats. Therefore, the hypoglycemic effect of PCD in diabetic rats may be partially explained by the recovery of the residual β -cells in the pancreas, restoring insulin secretion and enhancing insulin-mediated glucose uptake by target cells.

The skeletal muscle is responsible for around 80% of insulin-mediated glucose uptake, and therefore, it represents a major element in the maintenance of glucose homeostasis (19). Impairing GLUT4 translocation in the skeletal muscle is widely accepted as an insulin resistance mechanism, resulting in the consequent defect in insulin-stimulated glucose uptake (20). Several studies in skeletal muscles indicated that the amount of plasma membrane protein of GLUT4 is strongly correlated with glucose uptake (2). Therefore, translocation of GLUT4 to the cell surface could be part of the underlying mechanism responsible for PCD-mediated improvements in glucose homeostasis.

Existing evidence indicates that activation of PI3K promotes intracellular glucose uptake by GLUT4 translocation through insulin-dependent mechanisms

Table 1. Effect of PCD treatment on lipid profile in HF-STZ induced diabetic rats

Parameter	NC	D	D+PCD	D+MET
Triglycerides, mg/dL	120.2±5.3 ^a	235.2±10.1 ^b	159.4±10.4 ^c	149.5±12.3 ^c
Cholesterol, mg/dL	83.5±3.2 ^a	254.7±8.8 ^b	119.7±10.3 ^c	114.3±6.6 ^c
LDL, mg/dL	52.4±2.5 ^a	180.1±9.5 ^b	100.7±5.7 ^c	94.6±5.2 ^c

Abbreviations: PCD, dietary natural product prepared from *Cyphostemma digitatum*; HF-STZ, high-fat diet and streptozotocin; NC, normal control; D, diabetic; MET, metformin; SOD, superoxide dismutase; GSH, reduced glutathione; MDA, malonyl dialdehyde.

Data represent the means \pm SEM. Means with different superscript letters are significantly different from one another ($P < 0.05$).

Table 2. Effect of PCD treatment on oxidative stress in HF-STZ induced diabetic rats

Parameter	NC	D	D+PCD	D+MET
SOD U/mL	59.1±8.8 ^a	35.2±7.3 ^b	55.2±7.7 ^a	56.5±4.3 ^a
GSH μ mol/L	2.7±0.2 ^a	0.45±0.08 ^b	2.9±0.2 ^a	2.5±0.4 ^a
MDA, nmol/mL	4.4±1.5 ^a	12.1±2.5 ^b	4.7±1.7 ^a	4.0±1.2 ^a

Abbreviations: PCD, dietary natural product prepared from *Cyphostemma digitatum*; HF-STZ, high-fat diet and streptozotocin; NC, normal control; D, diabetic; MET, metformin; SOD, superoxide dismutase; GSH, reduced glutathione; MDA, malonyl dialdehyde.

Data represent the means \pm SEM. Means with different superscript letters are significantly different from one another ($P < 0.05$).

(2,21). The present study demonstrated that STZ injection and HF caused reductions in the GLUT4 levels and the efficiency of PI3K in the skeletal muscle of rats. These impairments in GLUT4 levels and PI3K activity were partially reversed by PCD treatment, suggesting that PCD might exert its hypoglycemic effect by accelerating the cellular uptake of glucose in the skeletal muscle via activating PI3K, leading to promoting GLUT4 translocation and therefore to the diminution of hyperglycemia.

Several studies have shown that oxidative stress contributes to the pathogenesis of diabetes mellitus (22). Due to impaired glucose metabolism, hyperglycemia causes various metabolic alterations, including oxidative stress (23), which can be defined as an imbalance between free radical formation and antioxidant defence capacity, subsequently causing lipid peroxidation (18,23). Oxidative stress has been shown to reduce insulin secretion and promote insulin resistance (18). In agreement with others (24), our current study showed that increased levels of MDA, one of the end products of lipid peroxidation, and decreased levels of GSH and SOD, an endogenous antioxidant defence system, are well documented in HF-STZ induced-diabetic rats (Table 2). However, treatment of diabetic rats with PCD significantly decreased the serum levels of MDA and increased the levels of GSH and SOD. The results obtained herein strongly suggest that PCD is beneficial as a protective agent against oxidative stress, as it significantly ameliorated HF-STZ-induced changes in the oxidative stress parameters, at least in part, via diminishing lipid peroxidation and enhancing free radical scavenger activity. This antioxidative property of PCD is certainly due to its chemical constituents, predominantly the presence of phenolic and flavonoid compounds, as was reported in phytochemical screening studies (6,8), which may exert an antidiabetic effect by increasing insulin sensitivity and/or insulin secretion and antioxidant potential.

It is well known that hyperlipidemia plays a significant role in diabetes and insulin resistance pathology (2,15). In the present study, the major changes in lipid profile, such as increased serum TG, TC and LDL, indicated significant dyslipidemia in diabetic rats. This outcome concurs with previously reported studies (25,26). These alterations in lipid profile may be attributed to increased intestinal absorption and cholesterol biosynthesis (27). An encouraging result of the present work is that 30 days of treatment with PCD showed a significant decrease in the TC, TG and LDL levels. These findings could be attributed to inhibiting the pathway of cholesterol synthesis, decreased intestinal absorption of lipids, improved glycemic control, and increased LDL removal by activating its receptors in hepatocytes (28,29).

Conclusion

Based on the findings, the present study has shown, for

the first time, that administration of the PCD significantly reduces hyperglycemia in HF-STZ-induced diabetic rats simply by restoring insulin production and facilitating skeletal muscle glucose uptake via activation of the PI3K-GLUT4 signalling pathways. The PCD used in the present study also significantly improves the serum lipid profile by lowering LDL, TC, and TG and correcting antioxidant biochemical parameters. These effects might be due to the presence of phenolic and flavonoid phytoconstituents. Therefore, PCD could represent a promising therapeutic agent to ameliorate and prevent type 2 diabetes.

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Authors' contributions

HA conceived and designed the study, drafted the manuscript, carried out the Western and biochemical studies. MA prepared PCD participated in the study design, helped to draft the manuscript, and performed the statistical analysis. All authors read and approved the final manuscript.

Conflict of Interests

All authors of this article certify that they have no conflict of interest.

Ethical considerations

All experimental procedures were carried out under the current ethical and care guidelines for the care of laboratory animals and were approved by the Animal Ethics Committee of Yarmouk University, Irbid, Jordan.

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